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Radial F-actin arrays precede new hypha formation in *Saprolegnia*: implications for establishing polar growth and regulating tip morphogenesis

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SUMMARY

The roles of cortical F-actin in initiating and regulating polarized cell expansion in the form of hyphal tip morphogenesis were investigated by analyzing long term effects of F-actin disruption by latrunculin B in the oomycete *Saprolegnia ferax*, and detecting localized changes in the cortical F-actin organization preceding hyphal formation. Tubular hyphal morphology was dependent on proper F-actin organization, since latrunculin induced dose-dependent actin disruption and corresponding changes in hyphal morphology and wall deposition. With long incubation times (1 to 3 hours), abundant subapical expansion occurred, the polar form of which was increasingly lost with increasing actin disruption, culminating in diffuse subapical expansion. These extreme effects were accompanied by disorganized cytoplasm, and novel reorganization of microtubules, characterized by star-burst asters. Upon removing latrunculin, hyperbranching produced abundant polar branches with normal F-actin organization throughout the colony. The results are consistent with F-actin regulating polar vesicle delivery and controlling vesicle fusion at the plasma membrane, and suggest that F-actin participates in establishing polar growth. To test this idea further, we utilized the hyperbranching growth form of *Saprolegnia*. Early during the recovery time, prior to multiple branch formation, radial arrays of filamentous F-actin were observed in regions with no detectable surface protrusion. Their locations were consistent with those of the numerous branches that formed with longer recovery times. Similar radial arrays preceded germ tube formation in asexual spores. The arrays were important for initiating polar growth since the spores lost their ability to polarize when the F-actin was disrupted with latrunculin, and increased isometrically in size rather than producing germ tubes. Therefore, F-actin participates in initiating tip formation in addition to its previously demonstrated participation in maintenance of hyphal tip growth. The cortical location and radial organization of the arrays suggest that they recruit and stabilize membrane-bound and cytosolic factors required to build a new tip.

Key words: Tip growth, Actin, Cell polarity, Spore germination, Branching

INTRODUCTION

Tip growth, wherein highly localized and polarized apical synthesis and secretion generate a characteristic tubular morphology, is the dominant growth form of an entire Kingdom, the Fungi (Heath, 1990; Gow, 1995). Fungi which grow by budding or fission show determinate tip growth, and have been used as model systems for genetic and molecular analyses of the process (reviewed by Drubin and Nelson, 1996; Mata and Nurse, 1997). Tip growth is also fundamental to plant reproduction and nutrition, and algal development, being the basis of growth of pollen tubes, root hairs, and algal rhizoids (Steer and Steer, 1989; Hepler et al., 1994; Kropf, 1994).

Regulation of this process is complex and not fully understood, due to the numerous cellular processes simultaneously required for polarized growth to occur. However, actin appears to be an important regulatory component of tip growth, situated high in the regulatory hierarchy. F-actin is involved in maintaining tip growth; it participates in the maintenance of tip shape (Jackson and Heath, 1990; Gupta and Heath, 1997), stabilization of the tip-localization of ion channels (Levina et al., 1994) and the transport and exocytosis of secretory vesicles (Novick and Botstein, 1985; McGoldrick et al., 1995; Ayscough et al., 1997; Mulholland et al., 1997). However, its precise mechanisms of action in these processes remain elusive.

F-actin is also involved in establishing tip growth. In the initial stages of fission and budding yeast elongation (Marks and Hyams, 1985; Adams and Pringle, 1984; Anderson and Soll, 1986), moss protonemata side branch formation (Quadar and Schnepf, 1989), and fucoid zygote germination (Brawley and Robinson, 1985; Kropf et al., 1989), F-actin accumulates in diverse patterns at the site of initiation. In algal zygotes, the actin accumulation is coincident with axis fixation, and has been postulated to recruit and anchor components needed for tip growth (Brawley and Robinson, 1985; Kropf et al., 1989; Kropf, 1992; Goodner and Quatrano, 1993). In *Saccharomyces*, numerous genetic and molecular analyses of

actin and actin-associated proteins demonstrate actin's involvement in bud initiation (reviewed by Drubin and Nelson, 1996) and in diverse fungi, F-actin disruption prevents germ tube emergence (Grove and Sweigard, 1980; Tucker et al., 1986; Barja et al., 1993; Harris et al., 1994). However, it is unclear exactly how the various reported organizations of actin function in these initiations. In addition, localized changes in actin organization have not been detected prior to branching or spore germination in filamentous fungal hyphae, complicating the issue of actin's function in establishing polar growth.

The oomycetes are protists that resemble filamentous fungi in their vegetative growth form, but are more closely related to fucoid algae (Bhattacharya and Druchl, 1988). They have proved to be a useful system for investigating actin's role in tip growth, as the large hyphae contain a complex and labile pattern of F-actin characteristic of growing cells (Heath, 1987; Temperli et al., 1990). Tips of growing hyphae contain an apical cap of fine F-actin filaments adjacent to the plasma membrane, extending back along the hyphae for approximately 30 μm, where they merge with the sub-apical pattern of cortical actin cables and plaques (Heath, 1987; Jackson and Heath, 1990; Kaminskyj and Heath, 1995). Previous work has indicated the importance of this population of actin in maintaining tip growth by supporting the weak apical wall from turgor-induced bursting (Jackson and Heath, 1990, 1993a; Gupta and Heath, 1997). However, actin's role in establishing tip growth is not clear, because the presence of abundant subapical actin cables and plaques, and the irregularity of branch initiation sites have made it difficult to detect polarized actin patterns preceding branch formation

In this report, we have explored the consequences of long term F-actin disruption in hyphae and spores of *Saprolegnia ferax*, and in the process discovered novel changes in F-actin organization preceding outgrowth, which allowed us to more clearly analyze actin's role in tip initiation.

MATERIALS AND METHODS

Hyphae of *Saprolegnia ferax* (Gruithuisen) Thuret (ATCC no. 36051) were grown overnight on dialysis tubing overlaying growth medium (OM; Heath and Greenwood, 1970) supplemented with 1.5% agar. Hyphal mats of approximately 1 cm × 1.5 cm were cut, and the hyphae were allowed to recover for 1 hour before subsequent handling.

The long term effects of F-actin disruption were investigated by incubating hyphae in 0.05, 0.1, 0.2, or 0.5 μg/ml latrunculin B plus 0.5% DMSO (Calbiochem, San Diego, CA) (Gupta and Heath, 1997) in OM for 1.5 or 3 hours, after which the corresponding hyphal morphology and F-actin organization were examined.

The F-actin organization in hyphae was visualized as described previously (Heath, 1987). Briefly, hyphae were fixed in 5% formaldehyde in 1,4-piperazinediethanesulfonic acid (Pipes) buffer, pH 7.0 (Sigma, St Louis, MO), with 100 μM MBS (*m*-maleimidobenzoyl *N*-hydroxysuccinimide ester; Molecular Probes, Eugene, OR) for 5 minutes, rinsed in buffer alone, stained for 5 minutes in 4.0 μM rhodamine-conjugated phalloidin (RP) (Sigma), rinsed in buffer, and mounted in Citifluor (Marivac, Ltd, Halifax, NS), an antifade agent.

The effects of latrunculin B on cell wall content and deposition were analyzed by incubating hyphae previously treated in 0.5 μg/ml latrunculin B for 1.5 or 3 hours, in 0.1% Aniline Blue (Fischer Scientific, Toronto, Ont.) in 60 mM Pipes buffer (pH 8.0) for 5 minutes. In order to determine any changes in the pattern of cell wall fibril deposition following 3 hours in latrunculin, isolated cell walls were shadow-cast. Briefly, hyphae treated with 0.5 μg/ml latrunculin for 3 hours were immersed in liquid nitrogen, crushed, and incubated in 1.5% sodium hypochlorite for 2 hours at 37°C. After washing the isolated walls with distilled water, drops of the cell wall preparation were dried on Formvar-coated mesh grids, and shadow cast with gold palladium alloy.

In order to determine whether latrunculin B-induced F-actin disruption altered other cytoskeletal components, microtubule organization was examined in hyphae treated with 0.5 μg/ml latrunculin B for 3 hours. Tubulin was immunolocalized according to the procedure of Kaminskyj and Heath (1994), with a few modifications. The fimbrin clot technique for immobilizing hyphal colonies was omitted; colonies were left on dialysis tubing for the duration of the procedure. Wall digestion and membrane permeabilization were carried out for 9 minutes each. During this procedure, nuclei were stained with mithramycin (Kaminskyj and Heath, 1994). To show that the alterations in microtubule organization in latrunculin B-treated hyphae were not the cause of the morphological changes, hyphae were preincubated in 2 μg/ml nocodazole (Heath, 1982) in OM for 1 hour, followed by co-incubation in fresh 2 μg/ml nocodazole and 0.5 μg/ml latrunculin B in OM for 3 hours. Tubulin was immunolocalized to confirm the disassembly of microtubules. The resulting hyphal morphology was compared to that induced in latrunculin B alone.

The extreme cytological and morphological effects of treating hyphae with 0.5 μg/ml latrunculin B for 3 hours were examined with transmission electron microscopy (EM), utilizing conventional fixation (Bachewich and Heath, 1997a).

To demonstrate that cells were still viable after 3 hours in 0.5 μg/ml latrunculin B, hyphae were placed in fresh OM without the drug for up to 40 minutes, during which they recovered polar growth in the form of hyperbranching throughout the colony. These hyphae provided a useful system for examining localized changes in F-actin preceding branch formation, due to the abundance of branches and recovery of F-actin organization. Shorter recovery times of 10-20 minutes, followed by fixation and staining with RP, were utilized for detection of such localized F-actin arrays.

To investigate the presence of localized F-actin arrays preceding outgrowth in a more normal situation for comparison, asexual spores (commonly referred to as cysts in this organism) were utilized. Cysts were induced according to the method of Holloway and Heath (1974). Briefly, hyphal colonies were incubated in dilute salts medium (DS) for 5 hours, with medium changes every 1 hour. After incubating the colonies at 4°C overnight, cysts were pelleted by centrifuging the medium for 15 minutes at 500 ***g***.

The F-actin organization associated with various stages of germination was observed by incubating cysts in 1 ml of OM in a 1 cm × 3.5 cm Petri dish on a rotary shaker for 30-60 minutes. Agitation interfered with cyst adherence to the Petri plate. Cysts were then fixed by adding 0.5 ml of 3× fixative (18% formaldehyde in 180 mM Pipes buffer, pH 7.0) directly to the Petri dish. After a 10 minute incubation, the fixed cysts were collected by centrifuging for 10 minutes at 500 ***g***. After removal of the fixative, approximately 25 μl of 8 μM RP was added to the cyst pellet, and incubated for 10 minutes. The stained cysts were then rinsed by addition of 500 μl Pipes buffer (60 mM, pH 7.0) alone, followed by a 10 minute centrifugation at 500 ***g***. Aliquots of the cysts were mounted in Citifluor, and examined for cysts containing localized changes in the cortical F-actin pattern, in the absence of any visible germ tube protrusion.

To determine the importance of the localized cortical F-actin organizations preceding germination for subsequent polar growth, cysts were incubated for 3 or 5 hours, or overnight, in OM as described above, with or without 0.5 μg/ml latrunculin B. The degree to which cysts were able to form polar germ tubes was compared.

Hyphae and cysts were analyzed with Nomarski differential interference contrast and epifluorescence optics, utilizing a Reichert

Polyvar microscope with a ×100, 1.32 NA objective. A G2 filter set was used for analyzing RP staining of F-actin, while a U1 filter set was used for visualizing mithramycin-stained nuclei and Aniline Blue-stained cell walls. Confocal microscopy (MRC600, Bio-Rad, Mississauga, Ont.) was also utilized to observe RP-stained actin, and immunolocalized tubulin, employing YHS and BHS filter sets, respectively, in combination with a ×60, 1.4 NA objective.

RESULTS

Latrunculin B induces F-actin disruption and corresponding changes in hyphal morphology and cell wall deposition

Numerous investigations have demonstrated the importance of actin in maintaining hyphal growth, including a recent study of the immediate effects of latrunculin B on *Saprolegnia* hyphae (Gupta and Heath, 1997). We have utilized a range of latrunculin B concentrations and longer incubation times to explore the consequences and feedback responses resulting from this form of growth inhibition.

Normal growing hyphae contain a cortical, fibrillar cap of F-actin in the apex, and cortical cables and plaques subapically (Heath, 1987) (Fig. 1A,B). The previously-reported latrunculin-induced disruptions of this organization (Gupta and Heath, 1997) persisted in a dose dependent manner after 1.5 hours in all concentrations of the drug. The fibrillar caps and cables were most sensitive, with thinning of the subapical cables, and shortening and thickening of the apical cap filaments in 0.05 μg/ml, while the plaques remained unchanged (Fig. 1C,D). The subapical cables were further reduced with 0.1 μg/ml, where only short, fine filaments could be seen amongst the plaques (Fig. 1E,F), and completely eliminated with 0.2 and 0.5 μg/ml (Fig. 1G-I). Apical caps were less abundant at higher concentrations, and when present, contained short, thick bundles of F-actin (Fig. 1G). At the higher concentrations, the overall intensity of actin staining was greatly reduced, plaques were smaller and replaced fibrils and cables in regions of diffuse subapical expansion (Fig. 1I). These characteristic patterns of disruption were maintained after 3 hours in all latrunculin B concentrations (data not shown).

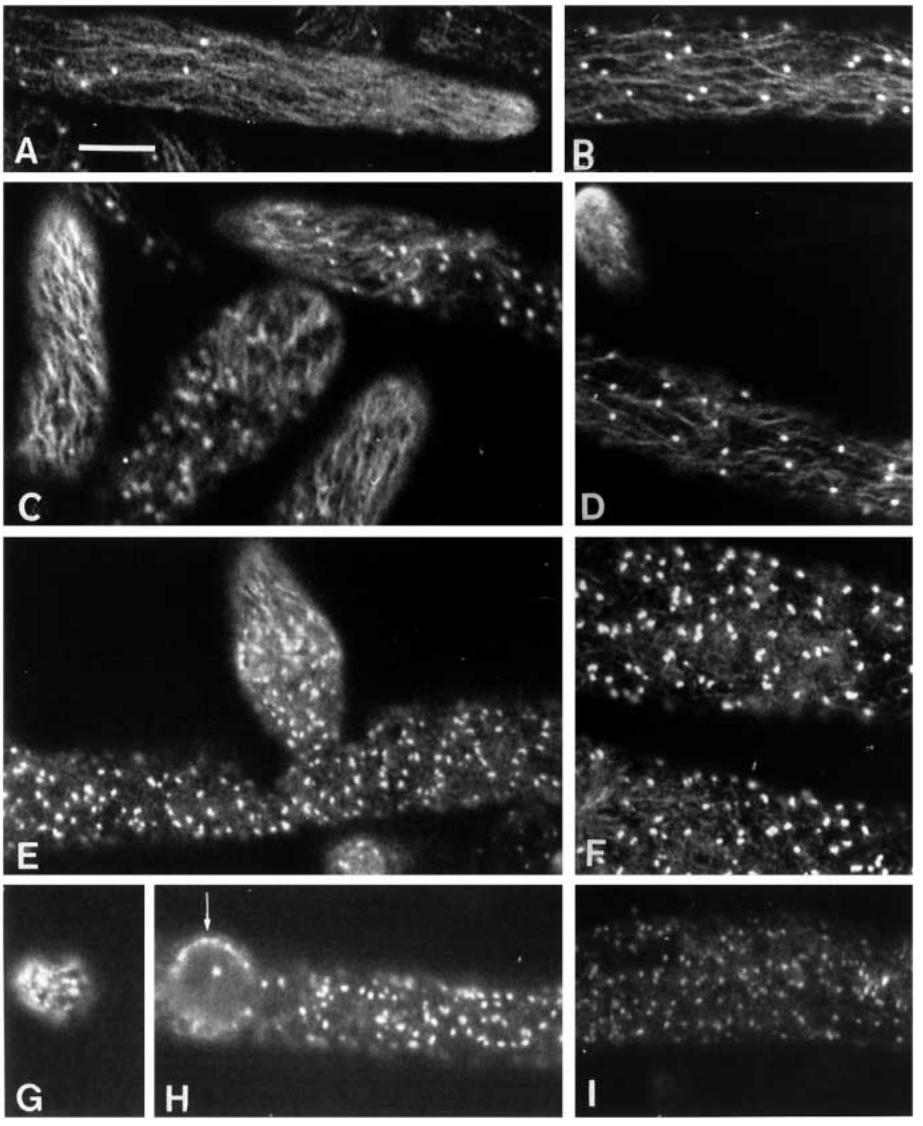

Fig. 1. Dose dependent disruptions of actin in hyphae exposed to various concentrations of latrunculin B for 1.5 hours. Actin in tips (A,C,E,G) and subapical regions (B,D,F,H,I) of hyphae exposed to 0.05 (C,D), 0.1 (E,F), 0.2 (G,H) and 0.5 (I) μg/ml became increasingly disrupted relative to the normal organization (A,B). (G) The cortical view of the same region indicated by the arrow in H. Note the progressive loss of filaments, and decrease in plaque size in the highest concentration (I). Bar, 10 μm.

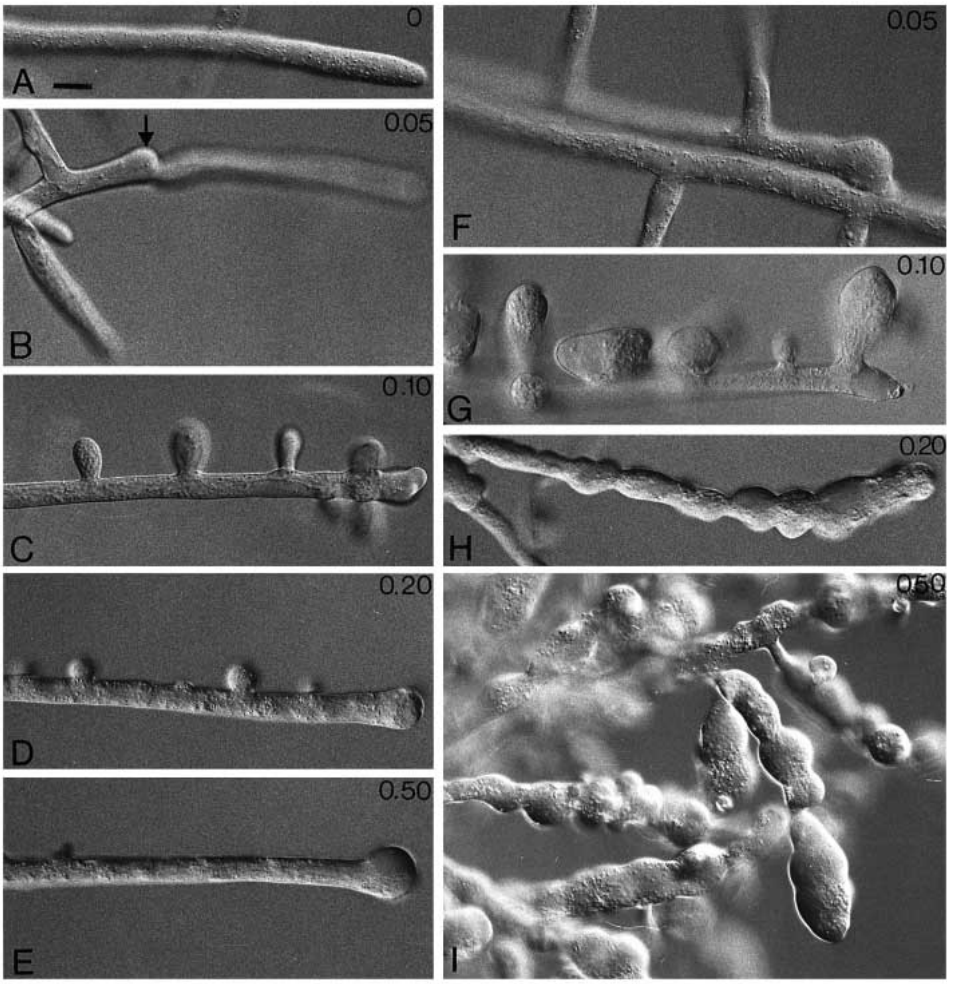

Fig. 2. Abnormal hyphal morphologies associated with latrunculin B-induced actin disruption. Morphologies resulting from 1.5 hours (B-E) and 3 hours (F-I) in the indicated concentrations of latrunculin B, compared to the normal morphology of hyphae (A). Hyphal tips initially swelled (arrow in B), but growth resumed after 1.5 hours. The resulting subapical outgrowths increasingly lost polarity with increasing latrunculin concentration. Note that subapical outgrowth was delayed in 0.5 μg/ml (E) relative to lower concentrations (C), but produced the most broad and diffuse extensions by 3 hours (I). Bar, 20 μm.

Concomitant with the F-actin disruptions were dose-dependent changes in hyphal morphology (Fig. 2). Initial application of 0.5 μg/ml latrunculin B was previously shown to induce a transient acceleration of hyphal growth, followed by growth inhibition and tip swelling (Gupta and Heath, 1997). All concentrations utilized here also induced initial tip swelling and growth inhibition, but growth resumed by 1.5 hours in the form of abundant subapical expansion. The morphology of the expansions was dose dependent, ranging from prolific tubular branches (hyperbranching) at the lower concentrations (Fig. 2B,C), to shorter and broader branches at intermediate concentrations (Fig. 2D). At the highest concentration there was considerably less subapical outgrowth (Fig. 2E). By three hours, the branches continued to expand normally at the lowest concentration (Fig. 2F), while they herniated at the intermediate concentration (Fig. 2G). At the higher concentrations, the branches were broader, due to more diffuse expansion (Fig. 2H). Abundant subapical expansion had occurred in the highest concentration by this time, dramatically increasing hyphal diameters in many regions (Fig. 2I).

The abnormal hyphal morphologies induced by latrunculin B involved both abnormal and normal cell wall synthesis. Wall thickness increased throughout the colony in the continued presence of latrunculin B, from the normal 30 nm (Fig. 3A) up to approximately 500 nm (Fig. 3B), and localized deposits formed throughout hyphae, appearing as random patches on the wall. The thickened walls were rich in fibrillar components, as shown by the stained images of prominent fibrils (Fig. 3B). The deposits were found in a variety of shapes, but typically formed a broad mound from which long, tubular extensions subsequently developed down into the cytoplasm (Fig. 3B-D). Entrapped membrane material was observed within some of these deposits (data not shown). Both the deposits and increases in wall thickness were visible by 30 minutes in 0.5 μg/ml latrunculin, but took longer to form in lower concentrations, not being present until 1 hour in 0.2 μg/ml, and 3 hours in 0.1 μg/ml.

Transmission EM demonstrated that many of the deposits contained a concentric pattern of microfibrils (Fig. 3C). To determine how the microfibrils were deposited, isolated cell walls were shadow cast. Only random wall fibril patterns were observed (data not shown), comparable to normal cell walls. However, wall deposits were difficult to identify in the preparations, suggesting that they had been solubilized during the isolation procedure.

The thickened cell wall and deposits initially stained yellow with Aniline Blue (Fig. 4C), a dye which stains newly synthesized plastic apical wall yellow, and does not react strongly with older, subapical regions, leaving them blue in part from autofluorescence (Fig. 4A,B). When hyphae were

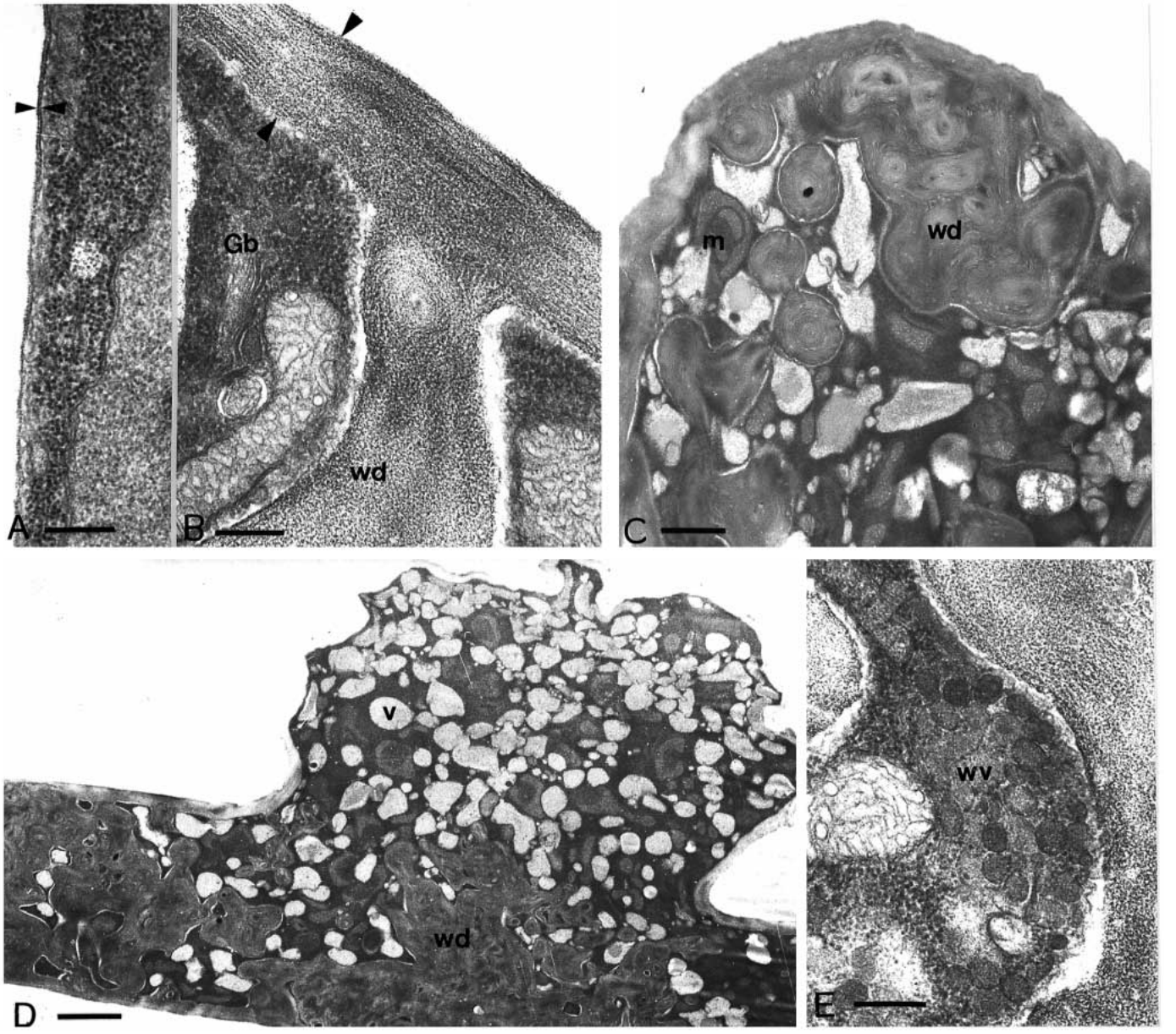

Fig. 3. Latrunculin B induces abnormal wall deposition. Thickened cell walls and wall deposits (wd) form after 3 hours in 0.5 μg/ml latrunculin B (B-E), relative to normal cell walls (arrowheads in A). Note the close proximity of the Golgi body (Gb) to the plasma membrane in (B), abnormal shape of mitochondria (m) in (C), which are normally tubular, abundant small vacuoles (v) in (D), and accumulations of wall vesicles (wv) near a thick region of wall in (E). Bars: 300 nm (A,B,E); 1.2 μm (C); 4 μm (D).

stained after longer periods of time in latrunculin (3 hours), the dye was excluded from many parts of the deposits and wall (Fig. 4D,E). Often, only the periphery of the tubular deposits stained yellow, presumably indicating that the central material was older, and no longer accessible to the dye (Fig. 4D). All of the tubules and deposits were extra-cytoplasmic, since they were enclosed by plasma membrane (data not shown).

The subapical expansions that occurred with longer incubation times in latrunculin B stained yellow with Aniline Blue (Fig. 4F), indicating that they were regions of new growth with wall properties similar to newly-synthesized walls. The wall regions flanking the subapical expansions were stained lightly or not at all (Fig. 4F).

Hyphae were able to adapt to lower concentrations of latrunculin B by resuming growth at the main tips of hyphae treated with 0.05 and 0.1 μg/ml by 1 hour (Fig. 2B,C), and from some wide branches produced in 0.2 μg/ml by three hours (data not shown). Although the recovered outgrowths were tubular in shape with 0.05 μg/ml, they were wider with the higher concentrations, containing abnormal F-actin caps as previously described.

F-actin disruption correlates with microtubule and organelle disorganization

Further examination of the extreme effects induced by 0.5 μg/ml latrunculin B for 3 hours revealed that the normal

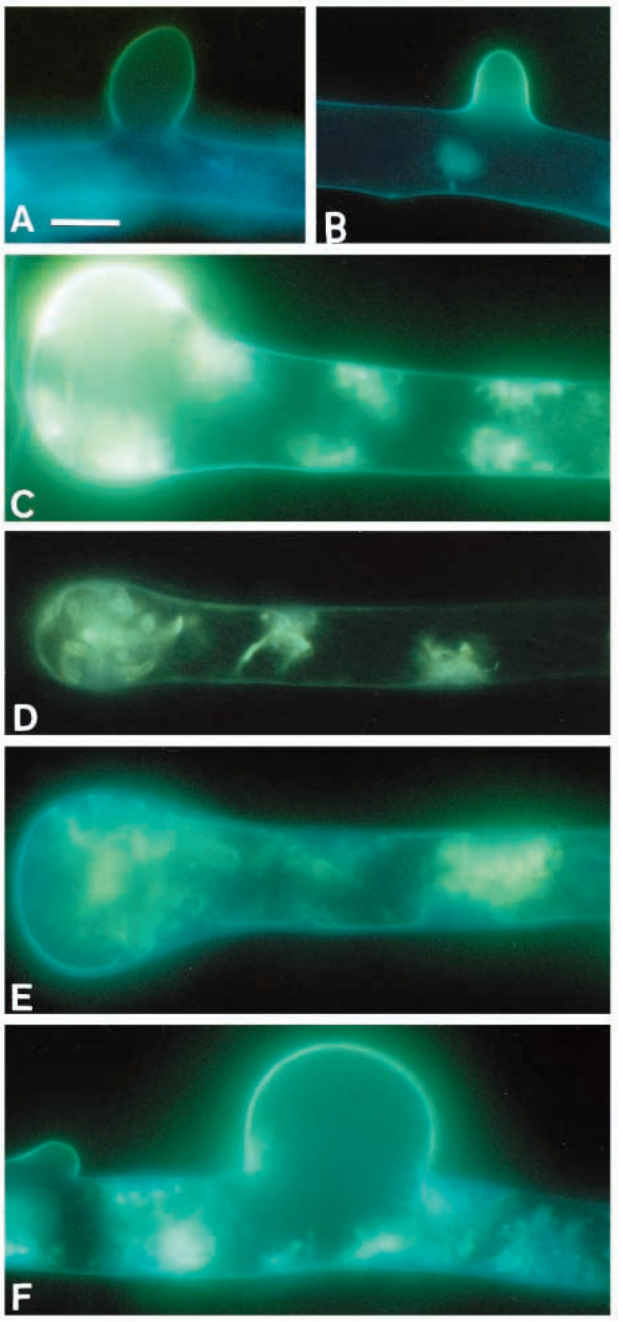

Fig. 4. Aniline Blue staining of newly-deposited wall. The cell walls of new, normal tips (A,B), and along the length and within deposits of hyphae incubated in 0.5 μg/ml latrunculin B for 1.5 hours (C) stain intensely yellow, indicating new wall synthesis. After 3 hours in latrunculin (D-F), only regions of wall deposits (D,E) and diffuse expansions (F) stain yellow, compared to the autofluorescent blue of older, nonexpanding walls. Bar, 10 μm.

longitudinal pattern of cytoplasmic microtubules (Fig. 5A; Kaminskyj and Heath, 1994), was often replaced with 'star-burst'-like patterns (Fig. 5B,C), each of which was associated with a nucleus (data not shown). These were most prevalent in the regions of hyphae that underwent diffuse expansion (Fig. 5B). The morphological and cell wall abnormalities described above were not attributable to this disorganization of microtubules, because their elimination with nocodazole, in the presence of latrunculin B, resulted in the same changes as latrunculin B alone (data not shown).

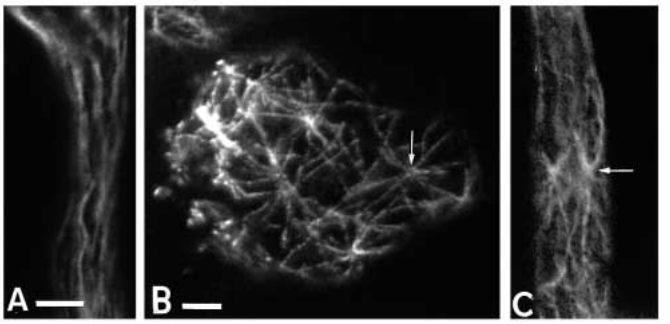

Fig. 5. Microtubule organization in hyphae treated with 0.5 μg/ml latrunculin B for 3 hours. (A) Normal longitudinal microtubules in a subapical region of a hypha. (B,C) Star burst-like patterns (arrows) amidst the predominantly longitudinal microtubules, most obvious in regions of diffuse expansion (B). Bars, 5 μm.

The latrunculin B-induced changes in the cytoskeleton also induced major alterations in organelle distributions. Vacuoles (Fig. 3D) and wall vesicles (Fig. 3E) were found throughout the cytoplasm, many mitochondria lost their normal elongated shape (Fig. 3C), and Golgi bodies apparently lost their normal concentration in the central cytoplasm, since they were frequently found in the cell periphery, often adjacent to the plasma membrane (Fig. 3B). Extensive analyses of the normal shape and distribution of such organelles have been previously reported (Heath et al., 1985; Heath and Kaminskyj, 1989).

Radial actin arrays precede hyphal branch formation and spore germination

The changes induced by 0.5 μg/ml latrunculin B for 3 hours were rapidly reversible in fresh OM without the drug. By 30 to 40 minutes, a high density of normal, tubular branches formed throughout the colony, some emerging immediately adjacent to each other (Fig. 6). They arose from all regions of hyphae, including the wide, diffusely-expanding regions. The branches contained F-actin caps similar to those of normal hyphal tips (data not shown), although the overall actin concentration was lower, allowing visualization of individual filaments. This production of abundant normal tips in subapical hyphal regions lacking the normal F-actin cables permitted us to investigate the organization of F-actin preceding new outgrowth.

When hyphae were allowed to recover for only 10 to 20 minutes, the cortical F-actin plaques were much brighter than before recovery, suggesting incorporation of more F-actin. However, the plaques were replaced with radial filamentous arrays of F-actin in localized regions (Fig. 7A,C,D,E). Optical sectioning showed that these arrays were essentially planar, adjacent to the plasma membrane, with no visible elevations in the hyphal surface. In several of these regions, DIC imaging revealed a corresponding radial organization in the cortical cytoplasm (Fig. 7B). The diameters of these arrays were variable, ranging from 9.3 μm to 20 μm. The average distance between adjacent radial arrays in several hyphae from a typical

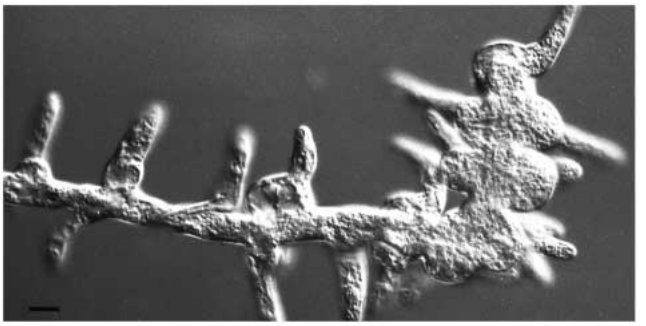

Fig. 6. Hyperbranching in hyphae recovering from latrunculin B. A hypha representative of a colony that was previously incubated in 0.5 μg/ml latrunculin B for 3 hours, followed by recovery in growth medium alone for 40 minutes, demonstrated abundant branch formation. Bar, 15 μm.

colony was 42±17 μm (n=11), with the smallest distance apart being 20 μm, similar to the distribution of subsequent branches (Fig. 6). Thus, the localization of the arrays was very similar to that of the branches which emerged after longer recovery times, suggesting the radial actin arrays initiate branch emergence. However, the inabilty to visualize cortical actin in living hyphae (Jackson and Heath, 1993b), and the lack of precise synchrony in branch emergence and irregular shapes of the treated hyphae preclude a more direct analysis of this relationship.

Similar radial arrays of F-actin occurred in pre-germinating cysts (Fig. 8). Cysts are produced in non-nutrient medium and remain spherical until germination upon transfer to nutrient medium. Non-germinating cysts contain peripheral plaques of F-actin (Heath and Harold, 1992), while germinated cysts contain F-actin plaques, filaments, and a cap in the tip of new germ tubes (Heath and Harold, 1992). Within asynchronous populations of germinating cysts, however, we detected cysts which still retained their spherical shape and contained random F-actin filaments amongst the plaques, while others displayed a more organized F-actin arrangement in the form of filamentous radial arrays converging to a single area on the cyst surface (Fig. 8B,C). These radial arrays, like those in the hyphae recovering from latrunculin B, replaced the plaques within the localized region in which they occurred. Upon germination, this array appeared to become the cap at the tip of the single germ tube. At very early stages of germ tube emergence, where the cyst surface was visibly protruded, the radial actin array was also protruded, lining the emerging germ tube (Fig. 8D).

In both hyphae and cysts, some radial actin arrays appeared to emanate from a central actin ring, ranging in diameter from 2.2 μm to 4.4 μm (data not shown). Such a ring was also present in some, but not all, tips of newly-formed branches. An equivalent depression in the cell surface was found in some of these regions with DIC imaging. However, the rings were not abundant, and through-focusing revealed that they were an illusion due to a localized inversion of the center of the radial actin arrays. The inversions may therefore be due to fixation-induced cytoplasmic movements (Kaminskyj et al., 1992; Doris and Steer 1996) which pull the apical actin subapically. The concomitant deformation of the cell wall in some hyphal regions could be a reflection of differential strength of newly-formed actin/wall adhesions (Kaminskyj and Heath, 1995; Henry et al., 1996; Bachewich and Heath, 1997b).

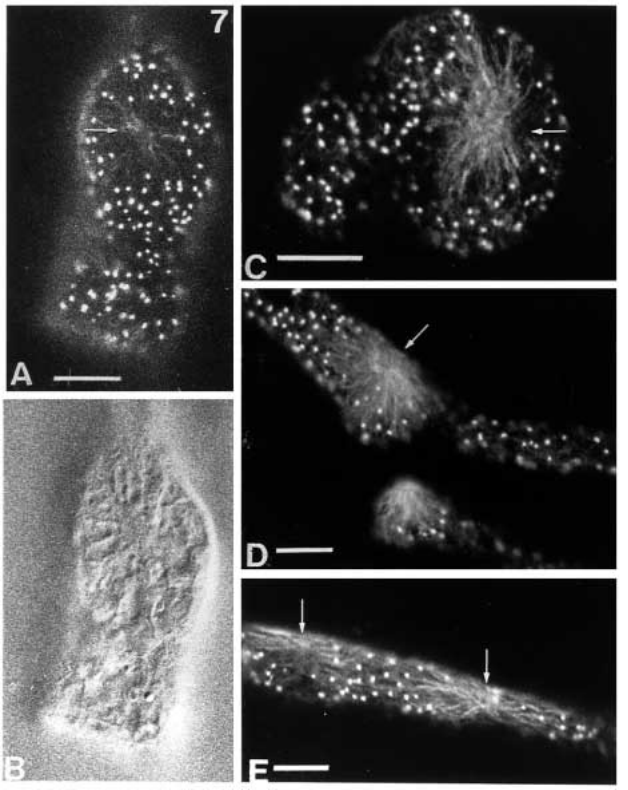

Fig. 7. The formation of radial actin patterns prior to branch emergence in hyphae recovering from 0.5 μg/ml latrunculin B. (A,B) Paired fluorescent RP (A) and differential interference contrast (DIC) (B) images of the same region of a hypha that recovered for 10 minutes in fresh medium alone following 3 hours in latrunculin B, followed by fixation and staining with RP. A radial filamentous array of actin in one plane is present amid the cortical plaques in A. A corresponding radial organization in the peripheral cytoplasm can be seen in B. (C-E) Confocal images of different hyphae under the same conditions as in A,B, demonstrating one (C,D) or two (E) radial F-actin arrays (arrows). These hyphae recovered for 15 minutes prior to fixation and staining. Note the similarity in distance between the two actin arrays in E and emergence of branches in Fig. 6. Also note the actin 'cap' of a newly emerged branch, resembling the radial actin array in the nonbranching hypha above it in D. Bars, 10 μm.

Latrunculin B-induced actin disruption prevents polarized spore germination

In order to demonstrate the importance of the radial F-actin arrays for initiating polar growth, cysts were germinated in 0.5 μg/ml latrunculin B. By 3 hours, the majority of control cysts had germinated and produced normal germ tubes (Fig. 9A), while germination in the presence of latrunculin B was rare (Fig. 9B). The diameter of many cysts had increased, which became more obvious after 5 hours (Fig. 9C). Some cysts apparently achieved some polarization, resulting in irregular shapes. The cysts contained abundant cortical actin plaques, but no caps (data not shown). Overnight incubation in

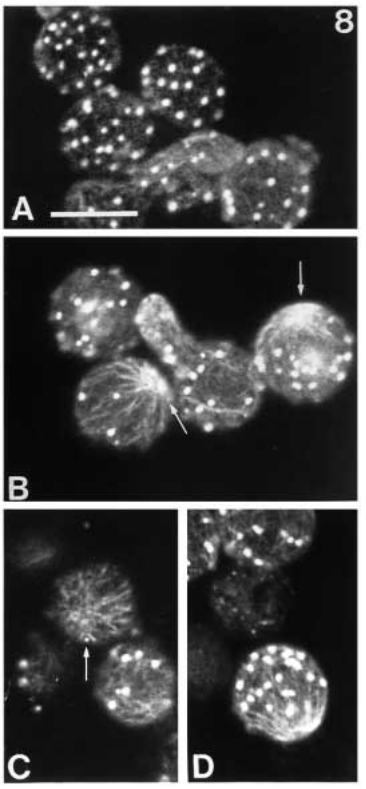

Fig. 8. Confocal images of peripheral actin patterns in cysts at different stages of development. Nongerminated cysts (A) predominantly contain only cortical plaques of actin, while germinated cysts (bottom cysts in A, middle cyst in B) contain actin plaques and cables. In several cysts that had not yet germinated, filamentous radial arrays of actin, converging to a central point, were visible (arrows in B,C). (D) A cyst showing early stages of germ tube emergence. Note the slight elevation in the cyst surface and corresponding radial actin array. All panels are at the same magnification as A. Bar, 10 μm.

latrunculin B produced giant cysts, up to 6 times their normal diameter (Fig. 9D). Wall deposition was clearly abnormal, as the walls were very thick (data not shown), and random patches with tubule-like extensions formed on the cell surface (Fig. 9D), resembling those produced in latrunculin B-treated hyphae. Thus, the cysts were able to grow and synthesize cell walls in the absence of normal actin organization, but their ability to polarize was severely impaired.

DISCUSSION

The effects of latrunculin B are mediated by F-actin

The present long term effects of latrunculin B were probably mediated by F-actin disruption because they resemble those induced by cytochalasins (Betina et al., 1972; Allen et al., 1980; Grove and Sweigard, 1980; Tucker et al., 1986; Shreurs et al., 1989), F-actin inhibitors with a different binding site (Cooper, 1987), and actin and actin-binding-protein mutations (Novick and Botstein, 1985; Gabriel and Kopecka, 1995; Goodson et al., 1996; Ayscough et al., 1997; Mulholland et al., 1997; McGoldrick et al., 1995). In addition, other mutants with disrupted actin organizations showed similar effects to latrunculin B (Bruno et al., 1996; Chiu et al., 1997). Furthermore, latrunculin A specifically disrupted actin in *Saccharomyces* at a concentration 200 times that which was effective in *Saprolegnia* (Ayscough et al., 1997). Although similar phenotypes are induced by alterations in fungal wall composition (Borgia and Dodge, 1992), the consensus of the above data indicates that the effects of latrunculin B on *Saprolegnia* are directly mediated by F-actin disruption.

Downstream functions of F-actin in establishing polar growth

This is the first demonstration of radial actin arrays forming prior to branch and germ tube emergence in tip-growing cells. Their occurrence in two very different cellular situations, one developmentally normal and one abnormal, indicates that the pattern is a prerequisite for establishing polarity. The location of the F-actin arrays adjacent to the plasma membrane and their radial organization suggest that they recruit and stabilize membrane-bound and cytosolic components, such as glucan synthases, ion channels, vesicle docking proteins, and vesicles, to generate a new tip (Brawley and Robinson, 1985; Kropf, 1992, 1994; Goodner and Quatrano, 1993; Drubin and Nelson, 1996; Ayscough et al., 1997). Their organizational similarity to the actin in growing hyphal tips suggests overlapping functions in establishing and maintaining growth.

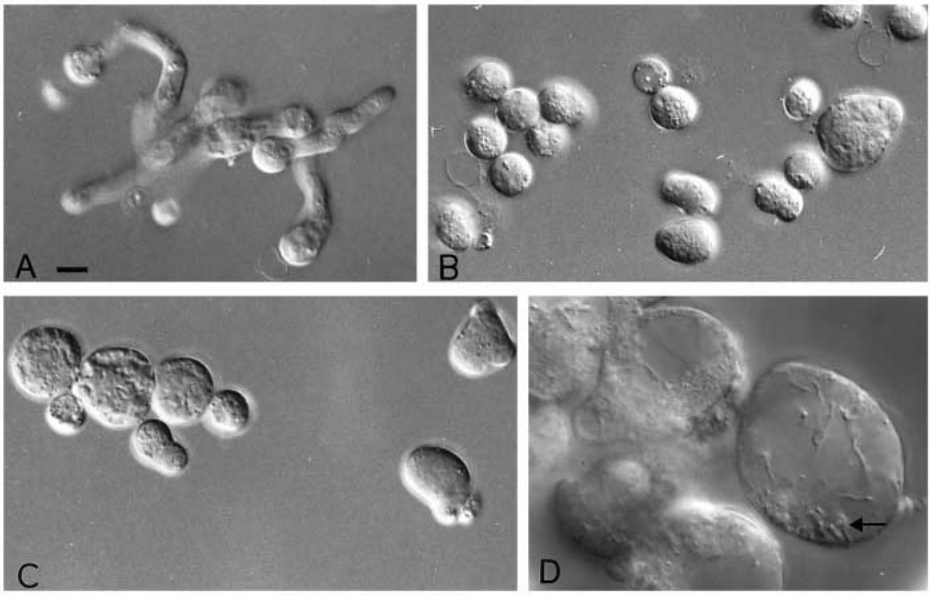

Fig. 9. Latrunculin B-induced actin disruption in cysts prevents polar germ tube formation. Cysts incubated in growth medium for 3 hours (A) form normal polar germ tubes, while those incubated in 0.5 μg/ml latrunculin B for 3 (B), 5 (C), or 24 (D) hours do not. Note the progressive increase in cyst diameter with longer incubation times in latrunculin, and the tubule-like wall deposits (arrow in D), resembling those in latrunculin B-treated hyphae. The odd shapes of several cysts indicate attempts to polarize (B,C). All panels are at the same magnification as A. Bar, 10 μm.

One of actin's functions in establishing polar growth is probably directing the apical delivery of vesicles containing cell wall precursors (Heath and Kaminskyj, 1989). This is supported by the production of subapical outgrowths upon inhibiting growth of the main tip, where vesicles presumably no longer delivered to the main tip become available for subapical branch formation. As F-actin was increasingly disrupted, the subapical outgrowths became increasingly broader, indicating that focused targeting was progressively lost. F-actin's role in apical vesicle delivery was also suggested by relationships between F-actin and vesicles in *Saprolegnia* (Heath and Kaminskyj, 1989), and vesicle accumulation and/or loss of polarized expansion in actin and actin-associated protein mutants of fungi (Novick and Botstein, 1985; Mulholland et al., 1997; Goodson et al., 1996; McGoldrick et al., 1995).

The membrane-associated F-actin is also likely to control vesicle fusion at the plasma membrane. In hyphae, vesicles are produced subapically and transported to the tip in the cortical cytoplasm (Heath and Kaminskyj, 1989), yet few fuse with the plasma membrane en route. The extensive subapical expansions following F-actin disruption indicate exposure of otherwise inhibited exocytotic sites, but some disorganized F-actin was still present. Similarily, F-actin depolymerization enhanced exocytosis in pancreatic acinar cells (Muallem et al., 1995), although a minimum amount was still needed. Actin is also required for localization of vesicles and vesicle docking proteins in *Saccharomyces* (Ayscough et al., 1997), and for polarized exocytosis in *Fucus* (Shaw and Quatrano, 1996). However, since subapical outgrowth took longer with increasing actin disruption in *Saprolegnia*, control of vesicle fusion probably involves mechanisms other than just regulating access to fusion sites.

Recruited wall vesicles are predicted to localize wall lytic enzymes, because diffuse wall lysis must have occurred to permit Aniline Blue staining of otherwise unstained, presumably inaccessible, subapical walls.

The localized movement and reorganization of membrane-bound actin, as shown by the production of the radial F-actin arrays, could induce changes in the localization and anchorage of several other factors, such as ion channels (Brawley and Robinson, 1985; Kropf, 1992, 1994; Kusimi and Sako, 1996; Goodner and Quatrano, 1993). F-actin has been shown to localize stretch-activated Ca^{2+} channels in growing tips of *Saprolegnia* (Levina et al., 1994). Co-localization of ion channels and wall lytic enzymes would lead to wall expansion, membrane stretching, activation of stretch-activated Ca^{2+} channels, tip-high gradients of Ca^{2+} (Garrill et al., 1993; Hyde and Heath, 1997) and an accelerating, self-sustaining, hyphal tip. Recruitment of components for tip production predicts that adjacent emerging branches would compete for resources, as shown by Grinberg and Heath (1997). Our observation of branches growing in closer proximity than observed by Grinberg and Heath (1997) suggests a greater pool of available resources in the unusual condition of hyperbranching.

Regulatory events upstream of radial F-actin array formation

Upstream regulation of the radial F-actin arrays probably involves minute, possibly stochastic, initiating cytoplasmic Ca^{2+} fluxes (Love et al., 1997). Trans plasma membrane ion fluxes (Kropf et al., 1983) and variations in Ca^{2+} concentration precede and influence the site of branch formation (Grinberg and Heath, 1997) and germ tube emergence (Hyde and Heath, 1995). These initiating fluxes could localize and activate a molecular polarity assembly pathway responsible for organizing F-actin, as described in *Saccharomyces* (Drubin and Nelson, 1996), forming the radial F-actin arrays. Since the radial arrays form in regions of plaque disassembly, the initiating Ca^{2+} flux could induce F-actin depolymerization, producing more polymerization-competent subunits for subsequent repolymerization mediated by hypothetical newly concentrated actin nucleating and regulating proteins. Increases in G-actin are likely during recovery from latrunculin B (Spector et al., 1989; Ayscough et al., 1997), which could contribute to the unusually high density of branches formed. A similar initial disassembly of F-actin must occur during normal branch formation, since the apical arrays replace the sub-apical cables and plaques (Heath, 1987). Since diverse organisms utilize some common mechanisms to generate polarity (Drubin and Nelson, 1996), the polarity organizing pathway in *Saccharomyces*, led by the GTPase Cdc42p (Ziman et al., 1993), may be involved.

Generality of radial F-actin arrays in tip establishment

The generality of the radial actin arrays reported here is not easily determined. Longitudinally-oriented actin arrays focused on the future site of septation in a fission yeast-like fungus (Butt and Heath, 1988), and radial arrays occurred in regenerating pollen tube protoplasts (Rutten and Derkson, 1990), but in the latter, the patterns were variable and did not predict the site of outgrowth. Other F-actin patterns preceding polar growth do not include radial organization, but it is neither clear if they represent the total population nor how the reported organizations function. For example, a membrane-associated ring of plaques and ring of filaments precede bud formation in *Saccharomyces* (Novick and Botstein, 1985) and branch formation in moss protomemata (Quadar and Schnepf, 1989), respectively, but their functions are unknown. Actin plaques in *Saccharomyces* correspond to membrane invaginations (Mulholland et al., 1994; Gabriel and Kopecka, 1995; Kaminskyj and Heath, 1996), but such invaginations are absent in hyphal tips of the many filamentous fungi that have been examined (Hoch and Staples, 1983; Heath et al., 1985; Roberson and Fuller, 1988; Bourett and Howard, 1991). However, there is evidence for cytoskeletal elements adjacent to the *Saccharomyces* plasma membrane (Kaminskyj and Heath, 1996), suggesting the presence of another population of cortical F-actin (possibly in a radial organization) undetected relative to the intensely stained plaques. Similar situations may exist in filamentous fungi, where actin plaques cluster in growing tips (Heath, 1990).

Adaptation to F-actin disruption

Hyphae adapted to latrunculin B, since they resumed growth in the presence of the drug. Hyperbranching is a common, possibly adaptive, response in fungi (Allen et al., 1980; Betina et al., 1972; Grove and Sweigard, 1980; Schreurs et al., 1989). Consistent with this, subapical hyperbranching occurred after 40 minutes in low concentrations of latrunculin, and after several hours (albeit in an apolar form), in higher

concentrations. Presumably vesicle fusion could not occur with extreme actin disruption (Ayscough et al., 1997; Mulholland et al., 1997), but some adaptation permitted diffuse vesicle fusion. Greater recovery was required for polarization. Consistent with this, replacing latrunculin B with fresh medium produced abundant polar outgrowths. Adaptation may depend on upregulation of actin synthesis, to compensate for the depletion of G-actin (Spector et al., 1989; Ayscough et al., 1997).

F-actin restrains plasma membrane-based wall synthetic enzymes?

In addition to its role in mediating exocytotic cell wall matrix synthesis discussed above, F-actin also seems to regulate wall fibril synthetic enzymes.

Subapical wall thickenings produced in latrunculin were rich in fibrillar components. Fungal mutants containing disrupted actin showed similar fibril-enriched wall thickenings (Chiu et al., 1997; Gabriel and Kopecka, 1995). Proliferation of fibrils in regions of the hyphae where little fibril synthesis normally occurs could be due to formation of new synthetic complexes, activation of normally quiescent ones or redistribution of apical ones. Disruption of the membrane-associated peripheral F-actin could permit configurational changes that reactivated inactive complexes. However, it is more likely that F-actin restrains wall synthetic complexes to the apex, as it does for membrane channel proteins (Levina et al., 1994). Membrane-bound proteins can diffuse at a rate of up to 2 μm/minute (Garrett and Grisham, 1995), providing ample capacity for apical enzyme complexes to diffuse to subapical regions and synthesize the many wall layers within the observed times. Delocalization of wall synthetic enzymes associated with actin disruption was previously suggested in fungi (Novick and Botstein, 1985; Gabriel and Kopecka, 1995; Goodson et al., 1996; Bruno et al., 1996; Mulholland et al., 1997; Chiu et al., 1997). It remains unclear how the wall synthetic enzyme activity becomes progressively restricted to localized patches, creating the wall deposits and their tubular extensions.

F-actin regulates cytoplasmic organization

The latrunculin B-induced disorganization of cytoplasm and organelles supports F-actin's role (Heath, 1990) in regulating the positioning and morphology of organelles such as mitochondria (Simon et al., 1995), Golgi bodies (Satiat-Jeunemaitre et al., 1996; Mulholland et al., 1997) and vacuoles (Hill et al., 1996). The concomitant alteration in microtubule and actin organization supports postulated F-actin and microtubules interactions (McKerracher and Heath, 1987; Collings et al., 1996). Since F-actin arrays remain normal in nocodazole-treated hyphae (G. D. Gupta and I. B. Heath, unpublished data), F-actin could stabilize microtubule organization. Since similar morphological effects result from incubation in latrunculin B with nocodazole, and latrunculin B alone, the microtubule rearrangements are a consequence of the latrunculin B effects.

Conclusion

The radial F-actin patterns described here are novel, and optimally arranged to recruit components to a specific site for tip production. They support F-actin's involvement in establishing polar growth, a feature possibly common to all tip-growing cells. However, differences with respect to the initial site selection process, upstream of the actin arrays, are anticipated in different species, since different organisms respond to diverse stimuli in different environments. Furthermore, in contrast to budding yeasts, pollen tubes, and algal rhizoids, hyphal organisms continuously form new subapical tips, suggesting some elements of the initiation and/or potential polarity organizing pathway are present throughout the hyphae.

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